

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

JONATHAN EDWARD LIGHTNER JOHN JOSEPH OKULEY

CASE NO.: BB-1043-A

COPY OF PAPERS

SERIAL NO.: 08/256.047

GROUP ART UNIT: 1803

ORIGINALLY FILED

FILED: OCTOBER 7,1994

EXAMINER: MCELWAIN

FOR: GENES FOR MICROSOMAL DELTA-12

FATTY ACID DESATURASES AND RELATED ENZYMES FROM PLANTS

Date: JANUARY 30, 1998

Assistant Commissioner for Patents Washington, D.C. 20231

Sir:

DECLARATION OF DR. ANTHONY JOHN KINNEY UNDER 37 CFR 1.132

- I, Anthony John Kinney declare as follows:
- 1. I am a citizen of the United Kingdom and am a permanent resident of the United States of America, residing in Wilmington, Delaware.
- 2. I received a B Sc. in biology from the University of Sussex in 1980 and a D. Phil. in biochemistry and cell biology from Oxford University in 1985.
- 3. I served as a research fellow in the Department of Food Science at Rutgers University, New Brunswick, N.J. 9/87-5/89.
- 4. I have been employed at E. I. du Pont de Nemours and Company (DuPont) since June, 1989 and presently work as a principal investigator for DuPont's agricultural products and am presently working on expression of storage oil genes.
 - 5. I have authored in excess of fifteen refereed articles in the field of biochemistry.
- 6. I have reviewed the above-identified case, and the Official Action for the subject case date? October 30, 1907. I understand that this declaration is being submitted to address ons of the pending claims under 35 USC §112 first paragraph or second paragraph and nerewith are a number of amino acid sequence alignments and phylogenetic tree Jopie Corps of source on plant C18.1 acyt-ACP dena-12 desaturases or acyt-ACP 12hydroxylases which were discussed with the Examiner at the interview held on December 0,

THEREBY CERTIFY THAT THIS PAPER IS BEING DEPOSITED WITH THE UNITED STATES PESTAL SERVICE WITH SELFICIENT PONTAGE AN HRST CLASS MAIL IN AN ÉNVELOPE ADDRESSED TO ASSEC OMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231, ON THIS DATE

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1997. All of these figures were prepared using the Megalign program from DNAStar, Inc., applying the comparison method of Hein (1990, *Meth. Enz.* 183:626-546).

The subject case discloses nucleic acid fragments encoding several plant C18:1 acyl-ACP delta-12 desaturases and a plant C18:1 12-hydroxylase which can be characterized as a delta-12 desaturase related enzyme. These nucleic acids encode proteins that form a distinct class of enzymes with regard to their amino acid sequence conservation.

Attachment I presents an alignment of the polypeptides set forth in SEQ ID NOS:2, 4, 6, 8, and 12; these are the predicted gene products from the nucleotide sequences set forth in SEQ ID NOS:1, 3, 5, 7, and 11. The shaded residues in the alignment indicate those residues that are conserved when compared with the *Arabidopsis* polypeptide (SEQ ID NO:2).

Attachment II is a phylogenetic tree depicting the calculated evolutionary/structural relationship of the sequences aligned in Attachment I. These figures clearly depict the close evolutionary/structural relationship among these proteins. Furthermore, it is also apparent that the 12-hydroxylase from castor is a member of this class of proteins. Within these alignments it can be seen that the delta-12 desaturase from soybean is more closely related to the hydroxylase from castor than it is to the other delta 12-desaturases disclosed in the subject specification. The sequences disclosed in the instant specification are also very closely conserved with other plant delta-12 desaturases.

Attachment III presents the amino acid sequence alignment of the previously mentioned polypeptides with those of other desaturases that have appeared in the public domain since the filing of the subject case. The additional sequences are for the proteins from *Brassica juncea*, parsley, potato, sunflower, and peanut (GenBank or EMBL accession numbers X91139, U86072, X92847, U91341, and AF030319, respectively). The corresponding phylogenetic tree for this expanded group of protein sequences is found (plotted on a logarithmic scale) in Attachment IV. These comparisons show clearly the relationship between these sequences, and convey graphically the expectation that any one of nucleic acid sequences set forth in SFD ID NO 1, 3, 5, 7, ct 1, may be used as a hybridization probe for genes encoding other plant delta-12 desaturases or desaturase related enzymes, such as 12-hydroxylases. This has been conformed in my laboratory.

Delta-12 desaturase or 12-hydroxylase protein sequences are structurally distinguishable from those of the next most closely related class, the delta-15 desaturases. Attachment V presents an amino acid sequence alignment of the previously described 12-

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desaturases with the sequences of plant delta-15 desaturases that were disclosed in WO 93/11245 (now pending USSN 08/244205). As suggested by the data within the specification for the subject case, it is relatively easy to identify delta 12-desaturases on the basis of their similarity, and moreover to distinguish them from the delta-15 desaturases due to their dissimilarity. The shading in this figure indicates those residue that are conserved relative to the delta-12 desaturase of *Arabidopsis*. Alignment of the 12-desaturases indicates that there is approximately 50% strict conservation or identity of amino acid residues across these proteins, however, they share only 30%-35% identity with any of the delta-15 desaturases. The phylogenetic tree depiction (Attachment VI) shows quite clearly that the two classes do not overlap. Rather, their members are separated by a highly statistically significant divergence, as is indicated by the scale along the bottom of the figure. Further support for this distinction comes from failed experimental attempts to use a gene encoding a plant delta-15 desaturase as a hybridization probe to identify and isolate genes that encode plant delta-12 desaturases.

The delta-12 desaturases are members of a group of proteins that catalyze various oxidat is of C18:1 fatty acyl groups at the C12-C13 bond. That is, other activities are known which catalyze mechanistically similar oxidations at this position, but do not necessarily produce a double bond in the product. However, it is not possible to say that these represent different classes of structures.

Disclosed in the subject case is the cDNA sequence from Ricinus communis (castor bean) encoding a 12-hydroxylase, which is a desaturase-related enzyme. The amino acid sequence of this protein is seen to fall cleanly within the "delta-12 desaturase" group when analyzed by a comparison method such as that of Hein (1990). Attachment V and VI also include within their presentations the placement of the hydroxylase from Lesquerella fendleri (WO 97/30582, SEQ ID NO:4). What is striking about this comparison is that the Lesquerella hydroxylase and the Arabidopsis delta-12 desaturase are more closely related to each other than they are to any other hydroxylase or desaturase included in the analysis. Not only are the proteins indistinguishable on the basis of their sequence, but it is now known that it may not be possible to distinguish them with regard to their function. Braun et all recently described results of experiments which sought to identify amino acid residues that disposed a profess to occur greather a desaturase or a hydroxylase (Broun et ai., in Physiology, But be mistry and Made at a Richery of Plant Lopits "Williams, J.P. et al., eds., Klawer Academic Publishers, Dordrecht, 1997, pages 342-344; a copy of which is enclosed herewith). During the course of their work, the authors expressed the "hydroxylase" from Lesqueretta in yeast under the control of an inducible promoter, and found that when

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compared to the uninduced control, the induced transformant produced both the C18:2 fatty acid, linoleic acid, and the 12-hydroxy form of oleic acid, ricinoleic acid. As it is known that yeast normally does not make detectable amounts of either of these oleic acid derivatives, it follows that the single protein from *Lesquerella* is responsible for the production of both products. Accordingly, it is both a "desaturase" and a "hydroxylase." These researchers then showed that mutation of only six amino acids (out of a total of 384 residues) was sufficient to convert this to a protein with properties closer to those of a "pure" desaturase. These results are depicted graphically in Figure 1 of the reference. Thus, it is apparent that this structural and functional class of proteins certainly includes more than enzymes that catalyze desaturation.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

DR. ANTHONY JOHN KINNEY

Date: 30 JAN 98



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Attachment I

Argnment Report of microfad2 MEG, using J. Hein method with PAM250 residue weight table.

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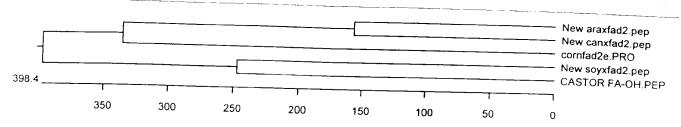
CASTOR FA-OH.PEP = castor 12-hydroxylase

New araxfad2.pep = Arabidopsis delta-12 desaturase

New canxfad2.pep = canola delta-12 desaturase

cornfad2e.PRO = corn delta-12 desaturase

Attachment II



New soyxfad.2pep = soybean delta-12 desaturase CASTOR FA-OH.PEP = castor 12-hydroxylase New araxfad2.pep = Arabidopsis delat-12 desaturase New canxfad2.pep = canola delta-12 desaturase cornfad2e.PRO = corn delta-12 desaturase

Attachment III

*at Report of Uspto2.MEG, using J. Hein method with PAM250 residue weight table. January 26, 1998 5-24 PM

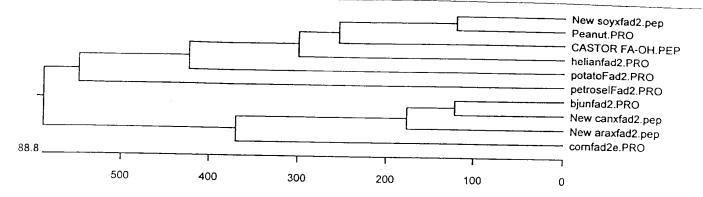
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LATUDREYGILNKVFHNITDTHVAHHLFSTMPHYHAMEA Majority 330 340 350 LATMDRDYCILINKVFHHITDTHVAHHLFSTMPHYHAMEA New soyxfad? pep MVTVDRDXGVLNXXXFHNIADTHVAHHLFATVDHXHAMEA CASTOR FA-OH PEP LATVDEDYSTENKVEHNITDTHVAHHLESTMEHXNAMEA New araxfad2 pep LATVDRDYSTANDGFHNTTDTHEAHHALFSATMBHYHAYBANNew canxtadl pep LATMDRDXGTUNRVFHNITDTHVAHHLFSTMFHXHAMBA, corntad2e PRO LATVDRDXGILNKVFHNITDTHVAHHUFSTMPHTHAMEV bjunfad2 PRO LATVDRDYGVLNRVFHHITDTTHVVHHLESTMPHXXAMEA helianfad2.PRO LATCDRDYGVLNKVRHNLTDTHVVHHLPSTMEHTTAATEA potatoFad2.PRO LATVDRDYCTENKAFHHITDTHVAHHLFSTMPHYHAMEA, Peanut PRO KAIKFILGDYYQFDGTPFYKAMWREAKECLYVEPDEGGS Majority 370 3.80 390 400 NAIKPILGE XX Q FOD D TOFF Y KALWREAR E.C.LY WE PEFEGTS New soyxfadd pep KAIKPIMGEXXRYDGTPFYFALWREAKBCLFVEEDEGAP CASTOR FA-OH PEP KAIKPILE GDYY OF DGTPWYVAMX REAKECTX VEREGO New araxfad: pep KAIKPILGEYYQRDGIPVVKAMWREAKECTYWEPDRQGE New canxfadi.pep KAIR PII G PANAH REDIP TIP VAKAT WREAG ENTIL WEELE D - - - cornfad2e.PRO KAIKRIIG DXXXQXDQXPWVKAMWREARBCIYYBERIR QGE bjunfad2.PRO KALEPVLGEXXREDKTPFXVAMWEEMKECLFVEQDDEXK helianfad2.PRO KAIKFILGDYYRRDDTPVVKAMWRBAKECLYXXXPEGDQ petroselfadî.PRO KAVKPLLGOYYQ COCTPIYK EMWREAKECLYVEK KDESSQ potatoFad2.FRO NAIKPILGDYYQFDGTPFYKALWREAKECLYYEFDDDGAS Peanut PFO FGVFWYNNKL - - -Majority KGVYWYENKY New soyxfadl pep Q G V F W Y E N K Y CASTOR FA-OH.PEP KGVYWYNNKL New araxfad2.pep KGVFWYNN New canxfad2.pep KGVFWYNKKF cornfad2e_PFO KGVFWYNNKL. bjunfad2.PRC - GVFWYFNRM - - N. helianfad2.PFC KGIFWYNNKL - - - petroselFad2.PRO KGVFWYKNKL potatoFad2.PF0 KGVYWYKNKF Peanut.PRD stion (lectration #2): Shade (with bright turquouse at 40% fill) residues that match araxfad2.pep exactly. New soymfad2.pep = soybean delta-12 desaturase CASTOR FA-GH.FEI = castor 12-hydroxylase New graxfad2.pep = Arabidopsis delta -12 desaturase New canxfad2.pep = canola delta-12 desaturase e na a kala 1986 – mena dikiterkili dalah male i funfado.PPO - Brassica funcea delta-12 desaturase 19 1 12 topo - Sunt lawar dal (3-1) dagaturada retroselfau2.FAu = paršely delta-,2 desaturask

Attachment IV



New soyxfad2 pep = soybean delta-12 desaturase CASTOR FA-OH PEP = castor 12-hydroxylase New araxfad2 pep = Arabidopsis delta-12 desaturase New canxfad2 pep = canola delta-12 desaturase cornfad2e PRO = corn delta-12 desaturase bjunfad2 PRO = Brassica juncea delta-12 desaturase helianfad2 PRO = sunflower delta-12 desaturase petroselFad2 PRO = parsley delta-12 desaturase potatoFad2 PRO = potato delta-12 desaturase

Attachment V

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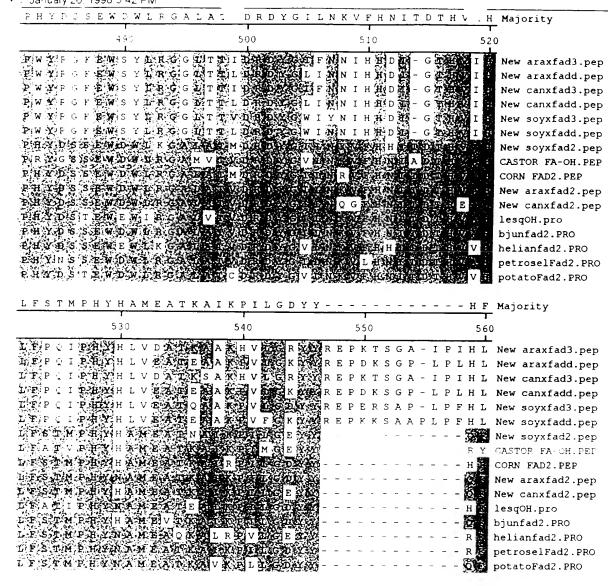
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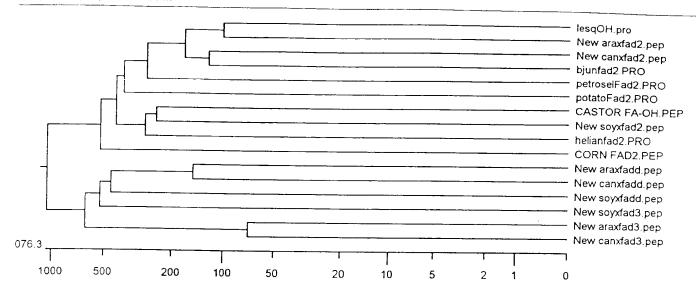
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D - B E A C HAR	PPNA	101		Lisadia - Ginana	Core New araxfad2.pep
DIG G FOA CHUE	H D M A	POLICE		I 500 4 6 3 4 5 6	Gay Col L New canxfad2.pep
D 6 E.A. s HAT	F P. H A	PAFKID		1 5 6 1 1 A	lesqOH.pro
FESTACHT	H PON A	PERMIT	3 T. A.	v S.58 G.11149-3	bjunfad2.PRO
DI-PFACHIY	V PTS	P M K N E	KRYCEV	M STOII GIVII	S F I helianfad2.PRO
E - P KAC H Y	D PKS	P HE S	AOLL	V Sadva O V Zara	S Y petroselFad2.PRO
D, - P F A C H Y	D PYG	BINNNR	F	ISSDA: GVIJG	C,Y L potatoFad2.PRO
			310-0000000		
RLAFAKGL	AWVL	C V Y G V P :	LLIVNM	FLVLITYLQH-	- T H P S Majority
PLAFAKGL	Т	CVYGVP	T	—————————————————————————————————————	
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3-3	Т	· v	460 Y I N F V M 1		480 G H D E K New araxfad3.pep
ALSFVF CP	1 450 1 A V L I I Q M L I	· grad i p	460 Y I I F V M 1 Y W N V M 1	470 WALDAVAXIHIHH WALDFVXXIHIHH	480 GHDEK New araxfad3.pep GHEDK New araxfadd.pep
ALSEVE CP	1 450 1 A V L I I Q M L I	Y V V V V V V V V V V V V V V V V V V V	460 Y I I F V M V Y W I N V M V Y I I F V M V	470 WADAV THEH WADFVEYTHEH WADFVEYTHEH	480 GHDEK New araxfad3.pep GHEDK New araxfadd.pep GHDDK New canxfad3.pep
ALSFVF CP	1 450 1 A V L I I Q M L I	V V V I I I	460 Y I I F V M V Y W I N V M V Y I I F V M V	470 WADAVANHHHH WADFVRAHHHHH WIDAVEXAHHHHH	480 G H D E K New araxfad3.pep G H E D K New araxfadd.pep G H D D K New canxfad3.pep G H E D K New canxfadd.pep
ALSFVF P CLNFTI P YLSFLV P CLNFVM P YLSFITSP	LAVLI IQMLI VTVLI		460 Y I I F V M 7 Y W F N V M 7 Y I C F V M 7 Y W F N V M 7 Y W F N V M 7	470 WADAVANHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH	480 GHDEK New araxfad3.pep GHEDK New araxfadd.pep GHDDK New canxfad3.pep GHEDK New canxfadd.pep GHEDK New canxfadd.pep
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ALSFVFCPPLSFLVCPPLSFLTSPLGFVMCPPRVMCP	450 LAVLI I Q M LI V T V LI M Q M LI L L V LI I C L LI V W L L		460 Y I II F V M Y Y W I N V M Y Y I T F V M Y Y W I N V M Y Y W F V M Y Y V I F V M Y	470 WADAVANHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH	480 GHDEK New araxfad3.pep GHEDK New araxfadd.pep GHEDK New canxfadd.pep GHEDK New canxfadd.pep GHEDK New soyxfad3.pep GHEDK New soyxfadd.pep - TAFF A New soyxfadd.pep
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A L S F V F G P C L N F T I G P Y L S F L V G P C L N F V M G P Y L S F I T S P J L G F V M G P R V A T L F G L C A T M A F G L K L A F G V	450 LAVLE I QULE V T V LE M Q V LE LL V LE V W L LO W W V V E	V I S I V I S	460 Y I I F V M V Y W I N V M V Y W I N V M V Y W F V M V Y W F V M V	470 WADAVIVIHIH WIDFVEYIHIH WEDFVEYIHIH WEDFVEYIHIH	480 G H D E K New araxfad3.pep G H E D K New araxfadd.pep G H E D K New canxfadd.pep G H E D K New canxfadd.pep G H E D K New soyxfad3.pep G H E D K New soyxfadd.pep - T G F A New soyxfad2.pep - T G F A CASTOR FA OH.PEF - CORN FAD2.PEP New araxfad2.pep
ALSFVFCP CLNFTIGP YLSFLVCP CLNFVMGP YLSFITSP JLGFVMGP RVATLFGL CATMACKGL KLAAAFGV	450 LAVLI IQVLI NOVLI LVLI CLLI VWLL AWVVI WWVVI	V G I P V I	460 YIIIFVM Y Y WIN VM Y Y IIFVM Y Y WIFVM Y	470 WADAVIVIHIH WIDFVEYIHIH WEDFVEYIHIH WEDFVEYIHIH	480 G H D E K New araxfad3.pep G H E D K New araxfadd.pep G H E D K New canxfadd.pep G H E D K New canxfadd.pep G H E D K New soyxfad3.pep G H E D K New soyxfadd.pep - TA F A New soyxfad2.pep - TA F A CASTOR FA OH.PEF - CORN FAD2.PEP New araxfad2.pep New canxfad2.pep
ALSFVFCP CLNFTIGP YLSFLVCP CLNFVMGP YLSFITSP JLGFVMGP RVATLFGL CATMACKGL KLAAAFGV RVAAAAOGW	450 LAVLE I QULE V T V LE M Q V LE LL V LE V W L LO W W V V E	V G I V I I V I I I V I I I V I I I I V I I I I V I I I I V I I I I V I	460 Y I I F V M V Y W I N V M V Y W I N V M V Y W F V M V Y W F V M V	470 WIDAVIVIHER WIDAVIVIHER WIDAVIVIHER WIDFVEYER	480 G H D E K New araxfad3.pep G H E D K New araxfadd.pep G H E D K New canxfadd.pep G H E D K New canxfadd.pep G H E D K New soyxfad3.pep G H E D K New soyxfadd.pep - TA F A New soyxfad2.pep - TA F A CASTOR FA OH.PEF - TA CORN FAD2.PEP New araxfad2.pep New canxfad2.pep - LE New canxfad2.pep - LE New canxfad2.pep
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A L S F V F C P C L N F T I C P Y L S F I V C P Y L S F I T S P R V A T L F C L A T M A F C L R V A A C C V R V A A C C V R V A A C C V R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L R V A M A F C L	450 LAVLI IQVLI IQVLI MQVLI LVVLI WWVVI WWVVI TVVLI TVVLI TVVLI	V G V I P V I P V A A V A V C G V C G V C G V C G C V C G C C C C	460 YIMFVMY YUNVMY YIMFVMY YUNVMY YUNVMY YUNVMY YUNVMY	470 WIDAVIVIHER WIDAVIVIHER WIDAVIVIHER WIDFVEYER	480 G H D E K New araxfad3.pep G H E D K New araxfadd.pep G H E D K New canxfadd.pep G H E D K New soyxfad3.pep G H E D K New soyxfadd.pep - T A CASTCE FA CH PEF - T CORN FAD2.PEP - New araxfad2.pep - T B C New canxfad2.pep
A L S F V F C P C L N F T I C P Y L S F L V C P C L N F V M C P Y L S F I T S P R V A T L F C L A T M A F C V R V A A C C V R V A A C C V R V A A C C V R V A A C C V R V A A C C V R V A A C C V R V A A C C C V R V A A C C C V R V A A C C C V R V A A C C C V R V A A C C C V R V A A C C C V R V A M A F C L	450 LAVLI IQVLI IQVLI MQVLI LVVLI WWVVI WWVVI TVVLI TVVLI TVVLI	V G V I P V I P V A A V A V C G V C G V C G V C G C V C G C C C C	460 YIIIFVM Y Y WIN VM Y Y IIIFVM Y Y WIFVM Y Y WIFVM Y Y WIFVM Y Y WIFVM Y Y V IFVM Y Y	470 WEDDAVTVIHHHH WEDDFVEYIHHHH WEDDFVEYIHHHH WEDDFVEYIHHHHH WEDTVIVILLE WEDTVILLE WE	480 G H D E K New araxfad3.pep G H E D K New araxfadd.pep G H E D K New canxfadd.pep G H E D K New canxfadd.pep G H E D K New soyxfad3.pep G H E D K New soyxfadd.pep - T A CASTOR FA OH.PEF - T B CORN FAD2.PEP New araxfad2.pep New canxfad2.pep - T B C CORN FAD2.PEP - T B C C CR



GTPVYKAMWREAL	ECVYVEPD	s	Majority
	T T T T T T T T T T T T T T T T T T T	500	-
570	580	590 60 — • • • • • • • • • • • • • • • • • • •	0
ESLVASIKKDHYVSDTG	DIVFY		New araxfad3.pep
. EILAKSIKEDHYVSDEG	EVVEYKAD	P	New araxfadd.pep
ert.	DIVFYTTPD	L Y V Y A S	New canxfad3.pep
	D V V X Y X A X	p	New canxfadd.pep
KYLIÇSMFQDHFVSDTG	DVV X YQT W	S L L -	New soyxfad3.pep
EIIPSFFTDHFVSDTG	DVVXYQTEE	S K I N	New soyxfadd.pep
ND TOP FORK ALL W REFINE	L L		New soyxfad2.pep
FYKALW R	L F	E G	CASTOR FA-OH.PEP
) P TOP V A K WIT W ROLL G	5.00 E		CORN FAD2.PEP
CALL STATE OF THE		- <i></i>	New araxfad2.pep
) GITP V V K AND W R		<u>Q</u>	New canxfad2.pep
OTTO THE STATE OF		T	lesqOH.pro
) G TO POW V K AND W ID		<u>-</u>	bjunfad2.PRO
K Table E XXX AND W REM	E C L F Q D	D -	helianfad2.PRO
D TAP V V K AND W +	T (E G	petroselFad2.PRO
CILE I X K E W W	EDIT KARAKET	E S	potatoFad2.PRO
- G K G V F W Y N N K L			Majority
610	620		
- D K S K I N			New araxfad3.pep
- N L Y G E V K V R A D			New araxfadd.pep
- D S K I N			New canxfad3.pep
- N L Y G E I K V T A E			New con fadd.pep
- L H S Q R D			New oyxfad3.pep
- G S S K L E			New s yxfadd.pep
S E KYG V Y W Y R W K Y			New soyxfad2.pep
PT CENER XX			CASTOR FA-OH PEP
- F K 6 7 F 7 N K 7 F			CORN FAD2.PEP
D.K KIG VIX F X N N			New araxfad2.pep
E R GG F 1			New canxfad2.pep
G X X G Y Y Y X X X			lesqOH.pro
EX KGVF FERN			bjunfad2.PRO
K G G X F X K M .	- N .		helianfad2.PRO
Q G K G I F X X	•		petroselFad2.PRO
QGKGVFWZK	•		potatoFad2.PRO

ation 'Decoration #1': Shade (with bright turquoise at 40% fill) residues that match araxfad2.pep exactly.

Attachment VI



New soyxfad2.pep = soybean delta-12 desaturase CASTOR FA-OH.PEP = castor 12-hydroxylase New araxfad2.pep = Arabidopsis delta-12 desaturase New canxfad2.pep = canola delta-12 desaturase CORN FAD2.PEP = corn delta-12 desaturase bjunfad2 PRO = Brassica juncea delta-12 desaturase helianfad2.PRO = sunflower delta-12 desaturase petroselFad2.PRO = parsley delta-12 desaturase potatoFad2.PRO = potato delta-12 desaturase lesqOH = Lesquerella fendleri 12-hydroxylase New araxfadd pep = Arabidopsis plastidic delta-15 desaturase New canxfadd.pep = canola plastidic delta-15 desaturase New soyxfadd = soybean plastidic delta-15 desaturase New soyxfad3 = soybean microsomal delta-15 desaturase New araxfad3 pep = Arabidopsis microsomal delta-15 desaturase New canxfad3.pep = canola microsomal delta-15 desaturase

Physiology, Biochemistry and Molecular Biology of Plant Lipids

Edited by

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Mobashsher Uddin Khan

and

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EXPRESSION OF CASTOR AND *L. FENDLERI* OLEATE 12-HYDROXYLASES IN TRANSGENIC PLANTS

Effects on lipid metabolism and inferences on structure-function relationships in fatty acid hydroxylases

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Introduction

Ricinoleic acid (D-12-hydroxyoctadec-cis-9-enoic acid), is an hydroxylated fatty acid which constitutes 85-90% of the seed fatty acids in castor bean plants (*Ricinus communis L.*). This unusual fatty acid is also one of a series of related Hydroxy Fatty Acids (Hi-As) produced in the seeds of *Lesquerella* species. In these species, which, like *A. thaliana* and rapeseed belong to the *Brassicacae* family, ricinoleic acid is generally a minor constituent. Major HFAs include densipolic (12-OH, 18:2 (3,9)), lesquerolic (14-OH, 20:1 (9)) and auricolic (14-OH, 20:2 (3,9)) acids.

In castor, where metabolism of HFAs has been studied in most detail, ricinoleic acid is synthesized in seeds on phosphatidyl choline, then very efficiently removed from membranes and transferred to the triacylglycerol pool (Bafor et al., 1991).

Recently, we have reported the isolation of a cDNA clone encoding the oleate 12- hydroxylase from castor (van de Loo et al., 1995). Constitutive expression of the hydroxylase cDNA in transgenic tobacco resulted in accumulation of low levels of ricinoleate in seed lipids, but not in leaves and roots. In order to further characterize metabolism of HEAs in transgenic plants, we have introduced this cDNA into 4 thaltana We report here how lipid metabolism is affected in transgenic plants.

Extraplastidual 6-6 desaturases and castor oleate 12- hydroxylase share a number of biochemical characteristics. Cloning of a cDNA encoding the castor hydroxylase has also confirmed that the two enzymes are closely related (van de Loo et al., 1995). Although reaction mechanisms are expected to be similar they lead to a lifework outcome. In order to investigate what structural components in these enzymes are applied to the latest the peak the order oreate 12-hydroxylase, from Lesquerella fendleri. Multiple comparisons of desaturase and hydroxylase sequences revealed key differences between the two categories of enzymes. We report here investigated as a their firestianal signofficance.

i. Expression of Castor and L. fendleri Hydroxylase Genes in transgenic A. thaliana

We designed degenerate primers based on the sequence of castor fatty acid hydroxylase CFah12 and used them to PCR-amplify cDNAs from *L. fendleri*. One such cDNA detected an abundant seed specific transcript on Northern blots of *L. fendleri* RNA. Its sequence had extensive similarity with the CFah12 gene. This cDNA was used to isolate a genomic clone which was introduced into *A. thaliana*.

Expression of the *L. fendleri* gene resulted in accumulation of HFAs in transgenic plants, up to 15% of seed fatty acids, thus establishing the gene encodes *L. fendleri* hydroxylase LFah12.

Transgenic plants expressing CFah12 under the control of a strong seed specific promoter were also obtained. In these plants, HFAs constitute up to 20% of the seed fatty acids. Seed fatty acid composition of *A. thaliana* plants expressing CFah12 or LFah12 is very similar, suggesting the two enzymes have comparable activities in transgenic plants.

Ricinoleic acid is only one of four HFAs produced in transgenic seeds, which also accumulate densipolic, lesquerolic and a small amount of auricolic acid. This suggests that *Arabidopsis* and related *Lesquerella* species metabolize ricinoleic acid in a similar way.

Expression of LFah12 under the control of the CaMV 35S promoter did not affect fatty acid composition of vegetative organs, even though hydroxylase activity could be detected. This implies either poor enzyme activity or efficient turnover of HFAs in non-seed tissues.

Accumulation of HFAs was accompanied with an increase in oleate levels and a concurrent decrease in 18:2 and 18:3, suggesting the oleate 12- desaturase is inhibited in transpenic plants expressing either gene

2. Investigations of Structure-Function Relationships in Hydroxylases and related Desaturases

We performed multiple comparisons between oleate 12- desaturases, CFah12 and LFah12, and identified six residues conserved among desaturases which differ in fatty acid hydroxylases. Using appropriate growth conditions (Covello and Reed, 1996), we were able to express LFah12 in yeast, under the control of the *GAL1* promoter. Yeast strains over-expressing the *Lesquerella* gene accumulated a small amount of ricinoleic acid. We could also detect small levels of 18:2, indicative of some desaturase activity of the enzyme in this context.

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In order to establish the functional significance of observed residue differences between desaturases and hydroxylases, we used site-directed mutagenesis to substitute desaturase residues for the corresponding residues in LFah12 at all six positions. In yeast strains expressing the mutant hydroxylase, ratios of 18:2 to ricinoleic acid levels were than 20 f. ld higher than in control atrains expressing the wild type gene (fig. 1). This result indicates that these residues are essential in LFah12 in determining the outcome of fatty acid oxidation.

Conclusion

We described here the isolation of a novel fatty acid hydroxylase from L. fendleri. We also presented some results from the analysis of transgenic A. thaliana plants expressing the castor and L. fendleri genes. We plan to use these transgenic plants to dissect mechanisms involved in removing HFAs from membranes, channeling them to storage lipids or breaking them down. We also hope to gain understanding of what controls such mechanisms.

We also reported here the critical role played by a small number of residues in controlling the outcome of fatty acid oxidation. Narrowing down on fewer residues will make it easier to rationalize structural differences between hydroxylases and desaturases, and understand how these differences affect reaction mechanisms.

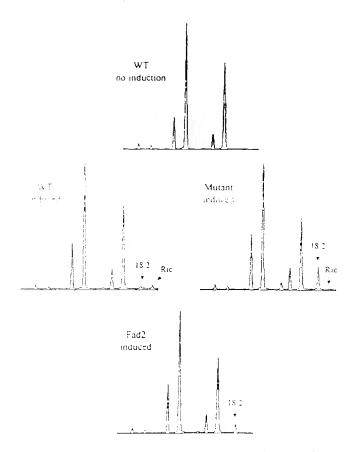


Figure 1: Ga: In a regram of fatty acid methyl esters from yeast strains expressing a variety pe or a mutant fatty acid hydroxylase from I stendleri.

Balor, M., Smith, M.A., Jonsson, L., Stobart, K. and Stymne, S. (1991) Ricinoleus and biocyotheus and master visit and exemply in microsomal preparations from developing castor-bean (Ricinus communis) endosperm. *Biochem. J.* 280, 507-514.

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Can de Episco, N. Bronn, P. Furner, S., Somerville, C.R. (1998) An oleate 12- hydrox-dasc from castor (Richney communis) pris a latty acyl desaturase homologue. *Proc. Natl. Acad. Sci. 1*, 84-92, 6743-6747.

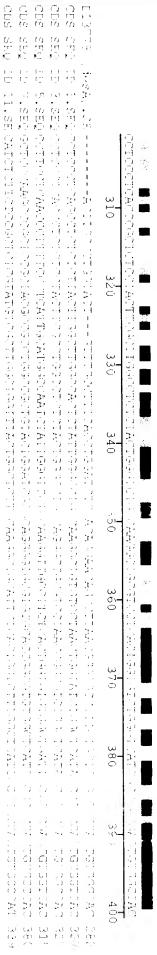
Alignment Workspace of fad2 Hein MEG, using J. Hein method with Weighted residue weight table.

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Alignment Workspace of fad2 Hein.MEG, using J. Hein method with Weighted residue weight table. Friday, April 04, 2003 1:46 PM

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Alignment Workspace of fad2 Hein.MEG, using J. Hein method with Weighted residue weight table. Friday, April 04, 2003 1:46 PM

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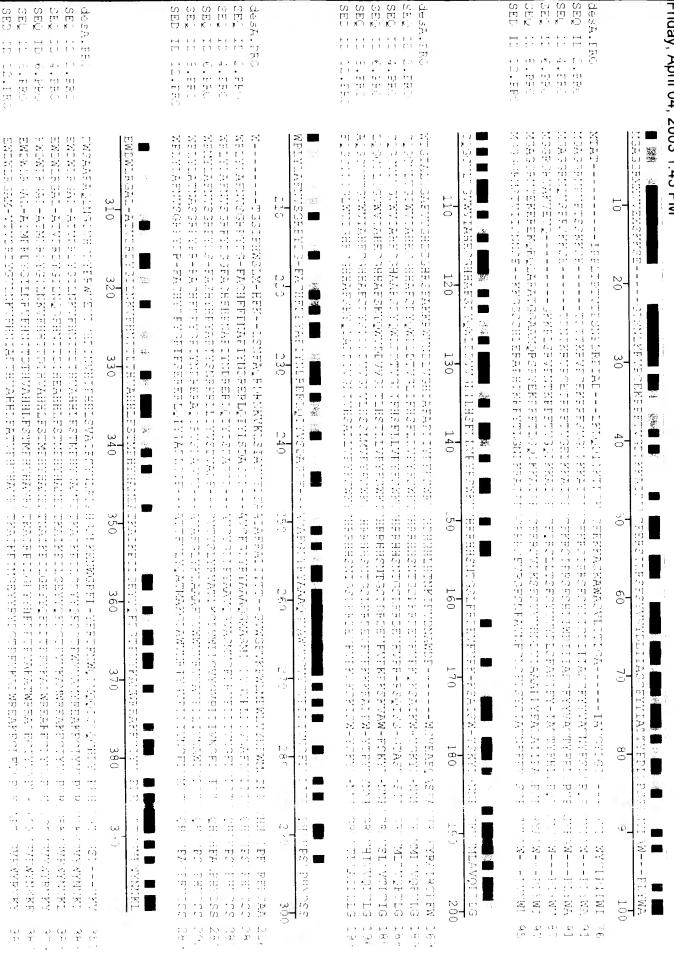
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Alignment Workspace of 1043-D suppl.:MEG, using Clustal method with PAM250 residue weight table

|Friday, April 04, 2003 1:43 PM



Percent Identity

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